

# *In Vivo* Recruitment of Hematopoietic Cells Using Stromal Cell–Derived Factor 1 Alpha–Loaded Heparinized Three-Dimensional Collagen Scaffolds

Bellinda A. Bladergroen, Ph.D.,<sup>1</sup> Bas Siebum, Ph.D.,<sup>2</sup> Kim G.C. Siebers-Vermeulen, B.Sc.,<sup>1</sup>  
Toin H. Van Kuppevelt, Ph.D.,<sup>3</sup> André A. Poot, Ph.D.,<sup>2</sup> Jan Feijen, Ph.D.,<sup>2</sup>  
Carl G. Figdor, Ph.D.,<sup>1</sup> and Ruurd Torensma, Ph.D.<sup>1</sup>

Implantable three-dimensional (3D) constructs to engineer tissue have great therapeutic potential in regenerative medicine and immunotherapy. However, autonomous recruitment of cells into the engineered scaffold *in vivo* is hampered by lack of attracting scaffolds. As a first step to engineering immune tissue, 3D collagen scaffolds were investigated for their ability to enhance *in vivo* recruitment and growth of various hematopoietic cells. Scaffolds containing immobilized heparin to trap the stem cell chemo-attractant stromal cell–derived factor 1 alpha (SDF1 $\alpha$ ) were implanted subcutaneously into C57Bl6 mice, and influx of cells was monitored using immunohistochemistry. Five weeks post-implantation, heparinized scaffolds were always populated by cells, but incorporating SDF1 $\alpha$  considerably stimulated recruitment of cells. SDF1 $\alpha$  could not exert this effect when the formation of a SDF1 $\alpha$  gradient was abrogated. Scaffolds were mainly populated by CD11b+ and CD11c+ myeloid cells and fibroblasts. One week after implantation, scaffolds harbored only low numbers of cells. Apparently, not all CXCR4-expressing cells, like large numbers of granulocytes, migrate into the scaffold, but retransplantation of a 1-week-old scaffold from a CD45.2<sup>+</sup> into a CD45.1<sup>+</sup> mouse yielded a scaffold harboring mainly CD45.2<sup>+</sup> cells after 5 weeks. These data confirm that only a few progenitor cells are recruited early after implantation. These cells then proliferate and differentiate along different lineages and determine the outcome after 5 weeks.

## Introduction

**T**ISSUE ENGINEERING USING implantable three-dimensional (3D) constructs with autologous cells emerges as a promising approach of cellular therapy for several diseases.<sup>1–3</sup> Mesenchymal and hematopoietic stem cells (HSCs) have great therapeutic potential in regenerative medicine, transplantation, and immunotherapy. Mechanisms exploited by nature's tissue engineering are the guidelines for artificial scaffolds used for *in vivo* tissue engineering.<sup>4</sup> The HSC is the best-characterized adult stem cell, and recent advances in purification techniques have improved the purity with which HSCs can be isolated from mouse and human bone marrow,<sup>5–7</sup> but despite progress in purification technology and the characteristic ability of stem cells to self-renew, huge expansion of HSCs *in vitro* has been unsuccessful. Moreover, *ex vivo* processing of cells is time- and cost-consuming. Limited expansion of undifferentiated adult stem cells has been a major obstacle for clinical applications of these cells.

*In vivo*, maintenance of HSCs and regulation of their proliferation and differentiation depends on their residence in special micro-environments, called stem cell niches.<sup>8–10</sup> Therefore, an alternative for *in vitro* expansion and processing might be *in vivo* recruitment to and growth of HSCs in a supportive scaffold with a micro-environment resembling the stem cell niche. Such a scaffold should allow local concentration, expansion, and manipulation in directing stem cell differentiation toward the cell types required therapeutically.

In bone marrow, stem cell niches comprise hematopoietic and stromal cells that create a complex but unique network of growth factors, chemokines, extracellular matrix, and adhesion molecules.<sup>8,9,11</sup> During homeostasis, HSCs are not confined to bone marrow niches; a small number of HSCs are released into the circulation, and circulating HSCs return to the bone marrow niche again. Furthermore, in response to signals from the periphery after injury, HSCs can move out of and into the bone marrow. Transplanted HSCs rapidly migrate to the bone marrow of the recipient.<sup>8,12</sup>

<sup>1</sup>Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands.

<sup>2</sup>Department of Polymer Chemistry and Biomaterials, University of Twente, Enschede, The Netherlands.

<sup>3</sup>Department of Matrix Biochemistry, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands.

The factor that plays a key role in the regulation of HSC homing and mobilization is the chemokine CXCL12, also known as stromal cell–derived factor 1 (SDF1) (reviewed in <sup>8</sup> and <sup>12</sup>). In bone marrow, osteoblasts, vascular endothelial cells, and fibroblasts produce SDF1. It functions as a chemo-attractant by forming a gradient between bone marrow and blood.<sup>13–15</sup> It has been shown that purified HSCs migrate specifically toward SDF1 and not toward other chemokines.<sup>16</sup> SDF1 exerts its effects by binding to the receptor CXCR4, which HSCs and hematopoietic progenitor cells (HPCs) express.<sup>17,18</sup> Although HSCs appeared to respond specifically to SDF1, SDF1 probably does not specifically control the trafficking of HSCs, because other cells express CXCR4 as well. For example, monocytes<sup>19</sup> and bone marrow stromal stem cells<sup>20</sup> also express CXCR4. Furthermore, the interaction between SDF1 and CXCR4 is essential for early B-cell development and the homing of end-stage B cells to the bone marrow.<sup>9,21</sup>

In bone marrow, glycosaminoglycans (GAGs) can bound and present SDF1.<sup>14,22</sup> Binding and tethering soluble factors such as growth factors and chemokines is one mechanism by which GAGs contribute to the formation of stem cell niches and regulate hematopoietic processes. Heparan sulfate, but also chondroitin sulfate and dermatan sulfate, which are present on the cell surface of bone marrow endothelial cells, guide migration of HPCs by binding and presenting SDF1. Moreover, *in vitro* studies suggest that endothelial heparan sulfate proteoglycans also control homing of HOCs by acting as direct adhesive ligands.<sup>23</sup>

As a first step in engineering immune tissue, 3D collagen scaffolds were used to recruit and support growth of hematopoietic cells in C57Bl6 mice. To improve the attracting and growth-supporting capacities of the scaffold, the effects of SDF1 $\alpha$  (SDF1 $\alpha$ ) and heparin on the population of the scaffolds by hematopoietic cells were investigated.

## Materials and Methods

### Mice

The animal experiments committee of the Radboud University Nijmegen Medical Centre (RUNMC) approved all animal experiments. C57Bl6 (N) mice were bred at the Central Animal Laboratory of the RUNMC. Adult C57Bl6.SJL-CD45.2 mice were purchased from Charles River Laboratories (L'Arbresle, France) and C57Bl6.SJL-CD45.1 mice were kindly provided by dr. R. van der Voort (Central Hematology Laboratory, RUNMC) but originated from Jackson Laboratory (Bar Harbor, ME) (stock no. 002014). CD45.1 and CD45.2 mice differ only slightly in their CD45 membrane molecule. CD45 is a marker molecule for leukocytes. Neither strain rejects cells from the other. Monoclonal antibodies to both CD45 variants are available, allowing the identification of the origin of the cells in the scaffold. All mice were housed in the specific pathogen-free unit of the Central Animal Laboratory at RUNMC.

### Materials

Insoluble type I collagen from bovine Achilles tendon and heparin from porcine intestinal mucosa (molecular weight (MW) 6,000–30,000, activity ~200 IU/mg) were purchased from Sigma (St. Louis, MO). N-(3-dimethylaminopropyl)-

N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS), and 2,4,6-trinitrobenzenesulfonic acid were obtained from Fluka (Buchs, Switzerland). Recombinant human SDF1 $\alpha$  was from R&D Systems (Minneapolis, MN).

Biotinylated monoclonal antibodies (mAbs) anti-CD11b mAb M1/70 (subtype rat immunoglobulin G2b (rIgG2b)); anti-CD45.1 mAb A20 (mouse IgG2a kappa (mIgG2a, $\kappa$ )); and anti-CD45.2 mAb 104, purified anti-CD16/CD32 mAb 2.4G2, purified rat IgG2a, $\kappa$  and IgG2b, $\kappa$ ; and purified hamster IgG, $\kappa$  (hIgG, $\kappa$ ) were purchased from BD Pharmingen (San Diego, CA). Biotinylated mAbs anti-CD11c mAb N418 (Armenian hamster IgG), anti-CD45R/B220 mAb RA3-6B2 (rIgG2a), anti-CD3 mAb 145-2C11 (hIgG), and anti-CD48 mAb HM48-1 (Armenian hIgG) were obtained from e-Bioscience (San Diego, CA), and biotinylated anti-CD41 mAb MW Reg30 (rIgG1) was from Serotec (Oxford, United Kingdom). Supernatant containing antibody ERT7 (rIgG2a) was a kind gift from dr. R. Mebius (Department of Molecular Cell Biology and Immunology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands), and anti-CD150 mAb 26D12 (rIgG1) was kindly provided by dr. R. de Waal Malefijt (DNAX, Palo Alto, CA). Anti-CD31 mAb MEC7.46 (rIgG2a) was from Hycult (Uden, the Netherlands), and biotinylated goat-anti-rat IgG was from Molecular Probes (Eugene, OR). Streptavidin-biotin-horseradish peroxidase complex (ABC<sup>PO</sup>), streptavidin-biotin-alkaline phosphatase complex (ABC<sup>AP</sup>), and levamisole were from Vector Laboratories, Inc. (Burlingame, CA). FastRed TR and naphthol AS-TR phosphate were obtained from Sigma, and optimal cutting temperature embedding matrix (Tissuetek) was from Cell Path (Newtown, Wales, United Kingdom).

### Preparation of collagen scaffolds

Collagen suspensions were prepared by swelling 2% (w/v) insoluble type I collagen in 0.5 M acetic acid at 4°C overnight. After dilution to 1% (w/v) collagen by addition of crushed ice, the suspension was dispersed for 4 min in a Philips Blender and subsequently homogenized at 4°C for 30 min using an Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany). The resulting slurry was filtrated through a 20-denier nylon stocking (with an average pore size of 30  $\mu$ m) and a 25- $\mu$ m Collector screen filter (Bellco, Feltham, United Kingdom) mounted in a disc filter holder. Entrapped air bubbles were removed by degassing at 0.1 mbar. The suspension was frozen overnight at –25°C in polystyrene trays and lyophilized.

Dried collagen matrices were chemically cross-linked with EDC and NHS.<sup>24</sup> Dried scaffolds were incubated in 0.05 M 2-(N-morpholino)ethanesulfonic acid buffer pH 5.5 for 30 min at room temperature. Subsequently, scaffolds were cross-linked using gentle shaking for 2 h in a 2-(N-morpholino)ethanesulfonic acid buffer containing 55.8 mM of EDC and 22.6 mM of NHS. The reaction was stopped by washing in 0.1 M sodium phosphate for 2 h, followed by rinsing three times for 30 min with demineralized water.

### Immobilization of heparin

Heparin was attached to EDC/NHS cross-linked collagen scaffolds for 2 h as described.<sup>24</sup> Control scaffolds were subjected to the same procedure except for the addition of heparin. The heparin content of cross-linked scaffolds was

determined using a colorimetric assay based on the binding of toluidine blue.<sup>25</sup>

### Characterization of scaffolds

After fixation with 2% glutaraldehyde (v/v) and 50% (v/v) osmium oxide the 3D structure of heparinized and non-heparinized scaffolds was evaluated using scanning electron microscopy.<sup>26</sup> The residual number of free primary amine groups of cross-linked and un-cross-linked collagen matrices was measured spectrophotometrically after reaction with trinitrobenzenesulfonic acid.<sup>27</sup> The shrinkage temperature ( $T_s$ ) was determined using differential scanning calorimetry.<sup>28</sup>

### Determination of SDF1 $\alpha$ binding and release

Recombinant human SDF1 $\alpha$  was labeled with <sup>125</sup>Iodine (<sup>125</sup>I) using Iodobeads as described previously (Pierce, Bioscience Etten-Leur, The Netherlands).<sup>29</sup> Collagen scaffolds (4 mg dry weight) with and without immobilized heparin were incubated overnight at 37°C in a solution of penicillin (200 U/mL) and streptomycin (200  $\mu$ g/mL). After being rinsed three times with phosphate buffered saline (PBS), the scaffolds were incubated overnight at room temperature in 1 mL of a solution of PBS with 2.22  $\mu$ g/mL of <sup>125</sup>I-labelled SDF1 $\alpha$ . Subsequently, scaffolds were rinsed three times with PBS, and radioactivity was measured using a Compugamma 1282  $\gamma$ -counter (LKB, Stockholm, Sweden).

To determine the release of SDF1 $\alpha$ , scaffolds that had been pre-absorbed with <sup>125</sup>I-labelled SDF1 $\alpha$  were incubated at 37°C in 5 mL of human plasma supplemented with 0.02% sodium azide. The medium was refreshed twice a week, and radioactivity in the medium was measured each time. After 2 weeks, the remaining radioactivity in the scaffolds was measured as well.

### Implantation of collagen scaffolds into C57Bl6 mice

Six-mm-diameter punches of heparinized and non-heparinized collagen scaffolds were sterilized by immersion in 70% ethanol for at least 6 h. After being washed 10 times

with sterile PBS, scaffolds were incubated in a solution of PBS containing 0.1% (w/v) bovine serum albumin (BSA) with or without 2  $\mu$ g/mL of recombinant human SDF1 $\alpha$  for 18 to 42 h at room temperature. In case scaffolds were subjected to a SDF1 $\alpha$ -washout, an 18-h incubation with SDF1 $\alpha$  in PBS/0.1% BSA was followed by an incubation for another 24 h at room temperature in PBS. All collagen punches were quickly rinsed three times in PBS and immediately used for implantation into C57Bl6(N) mice.

Six-mm punches were implanted subcutaneously via an incision in the back after the mice were anesthetized with isoflurane inhalation. Each mouse usually received two punches of the same type of scaffold. At 1 or 5 weeks post-implantation, scaffold punches were collected, immediately embedded in Tissuetek, and frozen in liquid nitrogen. Samples were stored at -80°C until further analysis.

To examine the recruitment of HPCs toward collagen scaffolds, a transplantation experiment with C57Bl6.SJL mice expressing different natural variants of CD45 was performed as follows. Eight-mm punches of heparinized collagen scaffolds incubated with or without 2  $\mu$ g/mL of recombinant human SDF 1 $\alpha$  (rhSDF1 $\alpha$ ) in PBS/0.1% BSA were quickly rinsed with PBS and immediately implanted subcutaneously (one punch per mouse) into CD45.2<sup>+</sup> C57Bl6.SJL mice as described above. At 1 week post-implantation, punches were collected and transplanted subcutaneously into C57Bl6.SJL mice expressing CD45.1 (one punch per mouse). After another 4 weeks in the CD45.1<sup>+</sup> mice, scaffold punches were collected and processed as described above.

### Immunohistochemistry

Sections (5  $\mu$ m thick) from frozen scaffolds were mounted on poly-L-lysine-coated slides and dried. Cell density and distribution were assessed on hematoxylin and eosin-stained sections. To identify specific cell populations, immunohistochemistry was performed with a panel of appropriate antibodies against various cell markers. A summary of the staining protocols is given in Table 1.

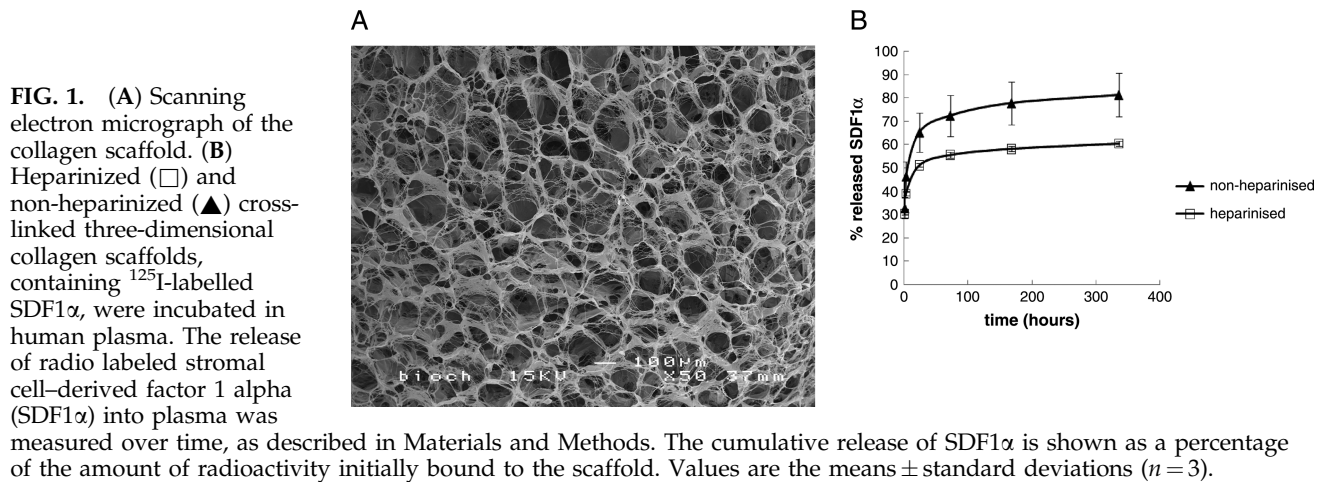
Sections were fixed with 4% paraformaldehyde for 30 min or in acetone for 5 min. Endogenous peroxidase activity was

TABLE 1. OVERVIEW OF THE STAINING PROCEDURES FOR VARIOUS CELL MARKERS ON FROZEN SECTIONS OF THREE-DIMENSIONAL COLLAGEN SCAFFOLDS

Step	Staining							
	CD11b Myeloid cells	CD11c Dendritic cells	CD3 T-cells	B220 B-cells	ERTR7 Fibroblasts	CD31 Endothelial cells	CD45.1 Leucocytes	CD45.2 Leucocytes
Fixation	PFA	PFA	acetone	PFA	acetone	acetone	acetone	acetone
Peroxidase block	+	+	+	+	-	-	-	-
Pre-incubation	PBS/BSA	PBS/BSA	PBS/BSA	PBS/BSA	NGS	NGS	-	-
Primary Ab	rIgG2b	haIgG	haIgG	rIgG2a	ERTR7	$\alpha$ CD31	CD16/CD32	CD16/CD32
Clone	-	-	-	-	-	MEC7.46	2.4G2	2.4G2
Concentration	5 $\mu$ g/mL	5 $\mu$ g/mL	5 $\mu$ g/mL	5 $\mu$ g/mL	1:5	10 $\mu$ g/mL	5 $\mu$ g/mL	5 $\mu$ g/mL
Secondary Ab	$\alpha$ CD11b <sup>bio</sup>	$\alpha$ CD11c <sup>bio</sup>	$\alpha$ CD3 <sup>bio</sup>	$\alpha$ B220 <sup>bio</sup>	G $\alpha$ R <sup>bio</sup>	G $\alpha$ R <sup>bio</sup>	$\alpha$ CD45.1 <sup>bio</sup>	$\alpha$ CD45.2 <sup>bio</sup>
Clone	M1/70	N418	145-2C11	RA3-6B2	-	-	A20	104
Concentration	5 $\mu$ g/mL <sup>a</sup>	5 $\mu$ g/mL	20 $\mu$ g/mL <sup>a</sup>	5 $\mu$ g/mL <sup>a</sup>	10 $\mu$ g/mL <sup>a</sup>	10 $\mu$ g/mL <sup>a</sup>	20 $\mu$ g/mL	20 $\mu$ g/mL
Detection	ABC <sup>PO</sup>	ABC <sup>PO</sup>	ABC <sup>PO</sup>	ABC <sup>PO</sup>	ABC <sup>AP</sup>	ABC <sup>AP</sup>	ABC <sup>AP</sup>	ABC <sup>AP</sup>
Visualization	DAB	DAB	DAB	DAB	FR	FR	FR	FR

Ab, antibody; ABC, avidin-biotin complex; AP, alkaline phosphatase; bio, biotinylated; BSA, bovine serum albumin; DAB, diaminobenzidine; FR, FastRed; NGS, normal goat serum; PFA, paraformaldehyde.

<sup>a</sup>Ab diluted in buffer containing 2% normal mouse serum.



blocked using incubation for 10 min with 0.3% (v/v) hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in PBS. Aspecific staining was blocked using incubation for 10 min with PBS/1% BSA or 20% (v/v) normal goat serum. To identify marker proteins, sections were sequentially incubated with the primary and secondary antibodies at a final concentration as denoted in Table 1. Incubation with each antibody was for 1 h at room temperature. The secondary antibody was biotinylated and diluted in PBS/1% BSA or PBS with 2% (v/v) normal mouse serum. Biotinylated secondary antibodies were detected using ABC<sup>PO</sup> or ABC<sup>AP</sup>. Bound antibodies were visualized using incubation with diaminobenzidine/ $\text{H}_2\text{O}_2$  or FastRed. Slides were counterstained with hematoxylin and mounted. Negative control slides were stained with IgG of the appropriate subclass.

CD150<sup>+</sup>CD41<sup>+</sup>CD48<sup>+</sup> hematopoietic stem cells were identified using triple staining as follows. After fixation with 4% paraformaldehyde for 30 min at room temperature, sections were preincubated for 30 min with 20% (v/v) normal donkey serum diluted in confocal laser scanning microscopy (CLSM) buffer (3% (w/v) BSA and 10 mM of glycine in PBS). Subsequently, sections were stained with anti-CD150 mAb 26D12 at 28  $\mu\text{g}/\text{mL}$  for 1 h at room temperature. CD150 was detected using incubation with APC-conjugated donkey-anti-rat, IgG diluted 1:200 in CLSM. After blocking with 10% (v/v) normal rat serum in CLSM, sections were then stained for 1 h with biotinylated anti-CD41 mAb MW-Reg30 (diluted 1:100) and CD48 mAb HM48-1 (20  $\mu\text{g}/\text{mL}$ ) in CLSM with 10% (v/v) normal rat serum. Biotinylated antibodies were visualized using incubation with Texas Red conjugated streptavidin, and sections were mounted. Fluorescence was analyzed using a Biorad confocal laser scanning microscope (Biorad, Hercules, CA).

## Results

### Cross-linking of heparin to collagen scaffolds improves their binding capacity for SDF1 $\alpha$

Three-dimensional collagen scaffolds consisted of a porous structure with an average pore size of 130  $\mu\text{m}$ , as evaluated from scanning electron micrographs (Fig. 1A). Cross-linking with EDC and NHS resulted in a decrease of free primary

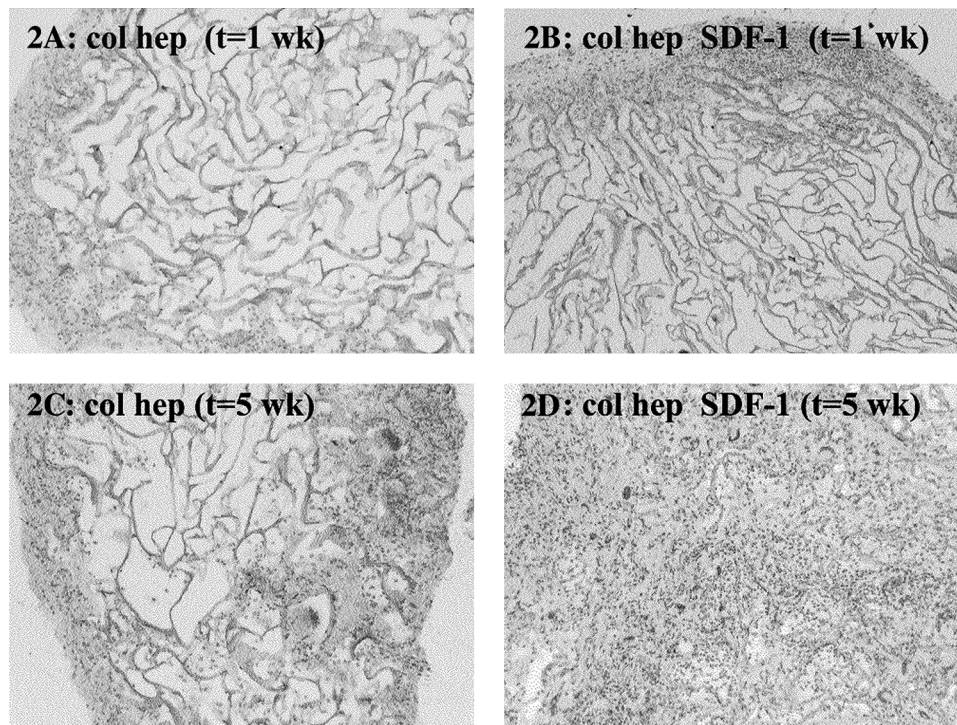
amino groups from 27 to 16 per 1000 amino acid residues. The shrinkage temperature increased from 59°C for uncross-linked collagen to 71°C for the cross-linked scaffolds. Thirty-four  $34 \pm 3 \mu\text{g}$  heparin per mg of collagen was attached to the scaffold, as measured according to the toluidine blue assay. Toluidine blue staining also showed that heparin had penetrated and had been attached to the collagen strains throughout the scaffold, including the central part (data not shown).

Upon overnight incubation in a solution with soluble SDF1 $\alpha$ , non-heparinized scaffolds bound approximately 50 ng of SDF1 $\alpha$  per mg of cross-linked collagen. Cross-linking heparin to collagen improved binding of SDF1 $\alpha$  to collagen scaffolds. Not only was the amount of SDF1 $\alpha$  bound to heparinized collagen higher (60 ng SDF1 $\alpha$  per mg of heparinized collagen), but the subsequent release from these scaffolds was also slower (Fig. 1B). Washout experiments showed that, from heparinized and non-heparinized collagen scaffolds, more than 50% of initially bound SDF1 $\alpha$  was released within 24 h after placing the scaffold in plasma (Fig. 1). After 24 h, the percentage of initially bound chemokine still present on cross-linked scaffolds without and with heparin was  $35 \pm 7\%$  and  $49 \pm 3\%$  ( $n=3$ ), respectively. The first phase of rapid release was followed by a second phase in which the rate of release gradually declined. After 2 weeks, 40% of initially bound SDF1 $\alpha$  remained present in heparinized collagen scaffolds. In non-heparinized scaffolds, this was only 20%. In the following *in vivo* experiments, heparinized collagen scaffolds were used, unless stated otherwise.

### SDF1 $\alpha$ stimulates recruitment of cells toward 3D heparin collagen scaffolds

After subcutaneous implantation, scaffolds always became populated by cells. One week after implantation, cells lying along the collagen fibers could be detected, although cell numbers were low (Fig. 2A). They were not distributed homogeneously throughout the scaffold, but most cells were present in a few spots at the periphery or in an area just around the scaffold. Five weeks after implantation, far more cells had populated the scaffold. By that time, not only had cell numbers increased, but their distribution had also





**FIG. 2.** Pre-incubation of three-dimensional heparin–collagen scaffolds with stromal cell–derived factor 1 alpha (SDF1 $\alpha$ ) markedly enhances their population by cells upon implantation. Hematoxylin and eosin staining of sections of heparin–collagen scaffolds incubated without (A, C) and with (B, D) SDF1 $\alpha$  before implantation. Scaffolds were collected 1 week (A, B) or 5 weeks (C, D) post-implantation. Original magnification:  $\times 100$ . Experiment was performed twice in duplicate.

changed (Fig. 2C). Now they were present almost throughout the scaffold, including the central part.

To achieve better recruitment of cells toward the scaffolds, the scaffolds were pre-incubated with SDF1 $\alpha$  just before implantation. Binding of SDF1 $\alpha$  to a scaffold resulted in a dramatic increase in the number of cells present 5 weeks after implantation (Fig. 2D). Whereas in the absence of SDF1 $\alpha$ , space between collagen fibers was rather empty, even 5 weeks after implantation, scaffolds with bound SDF1 $\alpha$  showed pores that were completely filled with cells and newly laid down extracellular matrix. SDF1 $\alpha$  could also exert its effects on cell recruitment when it was loosely absorbed by collagen, although the effect was somewhat less than in the presence of heparin. As can be seen in Figure 3, with or without heparin, scaffolds containing SDF1 $\alpha$  showed markedly more cells recruited than scaffolds without SDF1 $\alpha$ . Heparin itself had hardly any effect on the number of cells recruited to the scaffold. (Compare the scaffolds in Fig. 3D and 3E with those in 3A and 3B.)

SDF1 $\alpha$  could not exert its effect on cell recruitment any more when its ability to form a gradient was abrogated. If scaffolds containing SDF1 $\alpha$  were incubated in PBS, at least 50% of SDF1 $\alpha$  was released within 24 h from scaffolds with and without heparin (Fig. 1). If scaffolds subjected to such a 24-h SDF1 $\alpha$ -washout were then implanted *in vivo*, the level of cell recruitment was as low as toward scaffolds that had not been incubated with SDF1 $\alpha$  at all (Fig. 3).

#### *Hematopoietic and stromal cell types populated SDF1 $\alpha$ -treated heparinized collagen scaffolds*

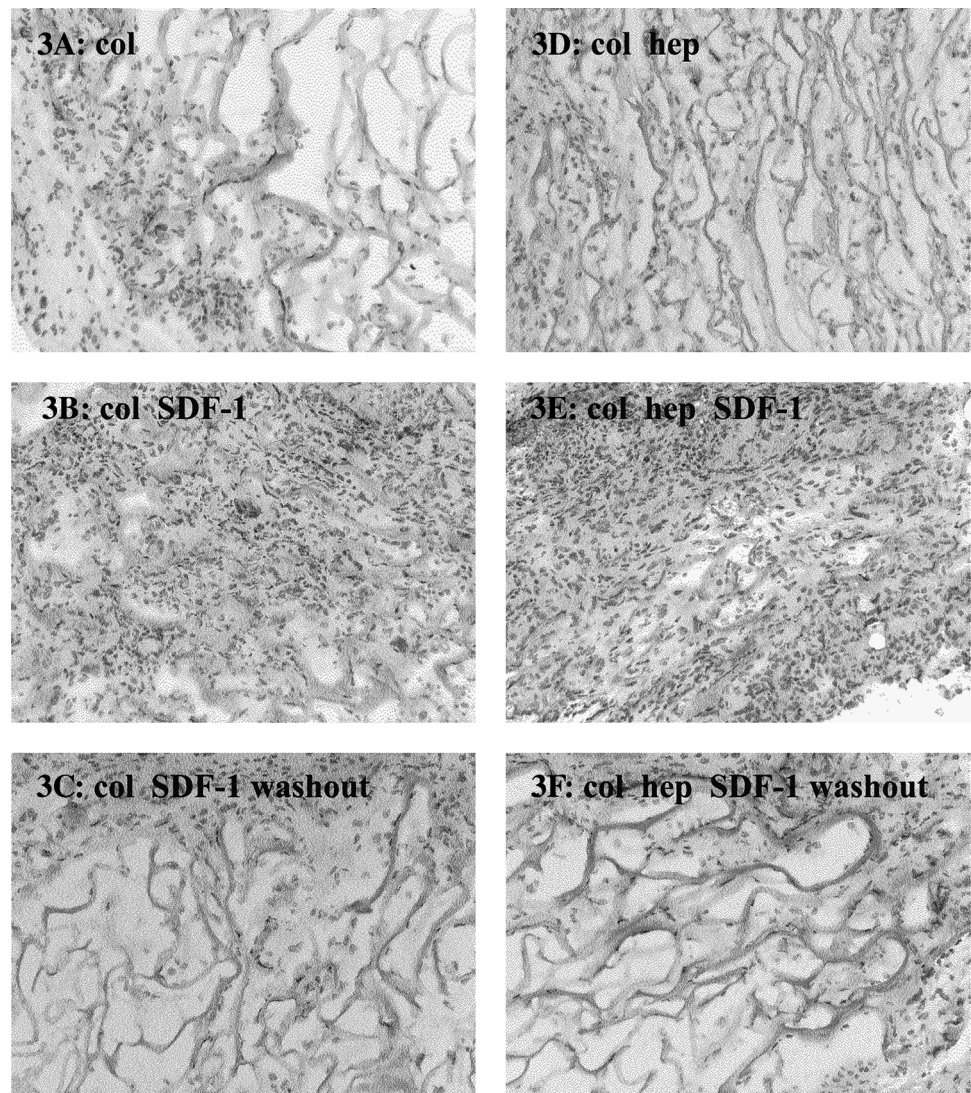
Five weeks after implantation, several cell types populated SDF1 $\alpha$ -soaked heparin collagen scaffolds (Fig. 4). Most he-

matopoietic cells were of the myeloid lineage, including CD11b $^{+}$  and CD11c $^{+}$  cells (Fig. 4A, B). CD11c $^{+}$  and CD11b $^{+}$  cells were scattered throughout the scaffold and were present as single cells lying along the collagen fibers or in small cell clusters. CD150 $^{+}$ CD41 $^{-}$ CD48 $^{-}$  HSCs could be detected in the scaffolds, although in low numbers (Fig. 4C). Moreover, hardly any CD45R/B220 $^{+}$  B-cells or CD3 $^{+}$  T-cells were found.

In addition to hematopoietic cells, stromal cells had been recruited to the scaffold. Expression of the fibroblast marker ERTR7 was observed mainly at the periphery of the scaffold (results not shown). Cells staining positive for CD31 indicated formation of small vessels (Fig. 4D). No staining was observed with isotype control mAbs (results not shown).

#### *Hematopoietic cells present after 5 weeks are mainly derived from cells recruited early after implantation*

Although at least a few CD150 $^{+}$ CD41 $^{-}$ CD48 $^{-}$  HSCs were found in some heparin collagen scaffolds 5 weeks after implantation, no such HSCs could be detected after 1 week. This probably reflects low numbers of these circulating HSCs. To examine the recruitment of stem or progenitor cells into these scaffolds, heparin collagen scaffolds were implanted into C57Bl6 mice expressing different natural variants of CD45. One week after implantation of scaffolds into CD45.2-expressing mice, most of the recruited CD45.2 $^{+}$  cells resided at the periphery of the scaffolds (Fig. 5A). When such a scaffold was transplanted from a CD45.2- to a CD45.1-expressing mouse, it contained considerably higher numbers of CD45.2-positive cells after 4 weeks that were distributed throughout the entire scaffold (Fig. 5C). Because these cells could only have been derived from the CD45.2-expressing



**FIG. 3.** The ability of stromal cell-derived factor 1 alpha (SDF1 $\alpha$ ) to form a gradient is essential to stimulate cell recruitment. Hematoxylin and eosin staining of sections of non-heparinized (A–C) and heparinized (D–F) collagen scaffolds incubated without (A, D) or with (B, E) SDF1 $\alpha$  or subjected to a SDF1 $\alpha$  washout (C, F) before implantation. All scaffolds were collected 5 weeks after implantation. Original magnification:  $\times 200$ . Experiment was performed twice in duplicate.

mouse, this indicates that CD45.2<sup>+</sup> stem or progenitor cells were already present at the time of retransplantation. During the 4 weeks that the scaffolds resided in the CD45.1-expressing mice, some CD45.1<sup>+</sup> cells were recruited (Fig. 5D), but their numbers were low, and they stayed mainly at the periphery of the scaffolds.

## Discussion

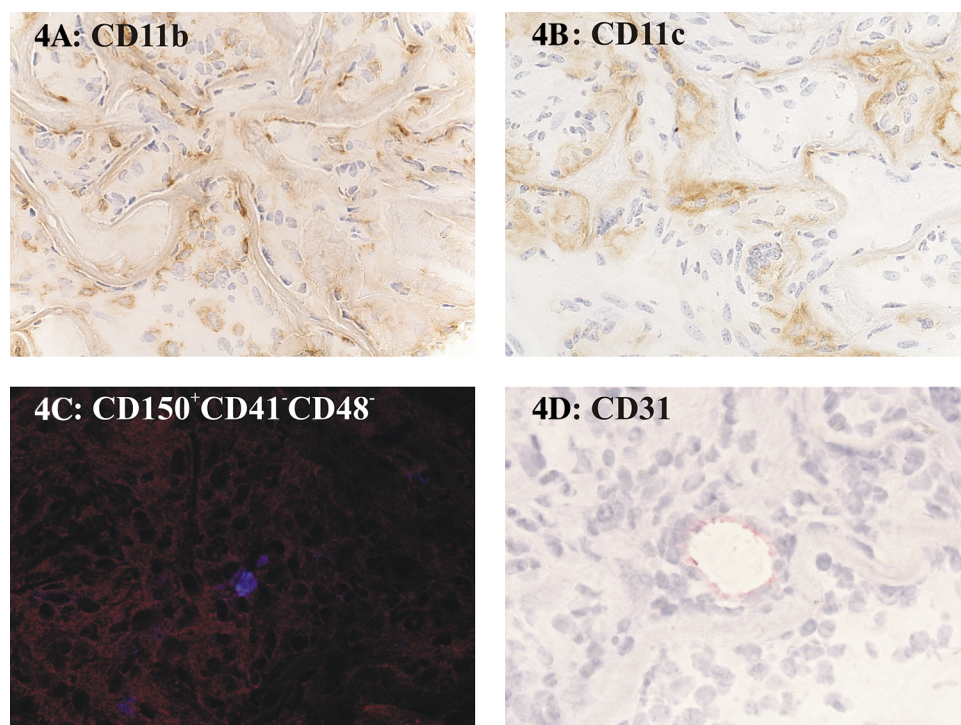
The present data demonstrate a novel approach to attracting circulating cells into a scaffold. This is a first step to engineer tissue without seeding cells in the scaffold before implantation. To promote cell attraction, we loaded the scaffolds with SDF, a chemokine involved in homing of circulating hematopoietic cells to the bone marrow. To create a gradient of SDF, scaffolds were loaded with the chemokine. Release kinetics indicated that the scaffold contained loosely absorbed SDF1 $\alpha$  that was released during the first 24 h and SDF1 $\alpha$  that was firmly bound by heparin *in vitro*. SDF1 $\alpha$  is released from heparinized 3D collagen scaffolds in two phases, with a rapid release of 50% of initially present SDF1 $\alpha$  in the first 24 h followed by a much slower release of an

additional 10% in the weeks thereafter (Fig. 1B). Assuming a similar release pattern *in vivo*, a gradient of SDF is present during the first 24 h after implantation.

Binding of SDF1 $\alpha$  to heparinized scaffolds was better than binding to bare collagen scaffolds. Heparin itself had hardly any effect on the number of cells recruited to collagen scaffolds. This is in contrast to what was expected, because heparin and heparan sulfate proteoglycans (HSPGs) have been reported to contribute significantly to formation of niches and play a dominant role in the regulation of hematopoiesis,<sup>30</sup> although in the latter situation, HSPGs carry trapped growth factors. It has been hypothesized that HSPGs contribute to homing of HPCs to bone marrow,<sup>14</sup> are adhesive ligands for adhesion molecules such as integrin CD11b/CD18,<sup>31</sup> and bind many chemokines and growth factors.<sup>22,32</sup>

The putative gradient of SDF1 $\alpha$  formed during the first phase of release appeared to be essential for cell recruitment (Fig. 2), because abrogation of gradient formation by SDF1 $\alpha$  washout resulted in markedly fewer cells present in scaffolds at 5 weeks after implantation (Fig. 3C, F). It has been reported that SDF1 $\alpha$  can mediate migration of T-cells without the formation of a gradient when endothelial cells present





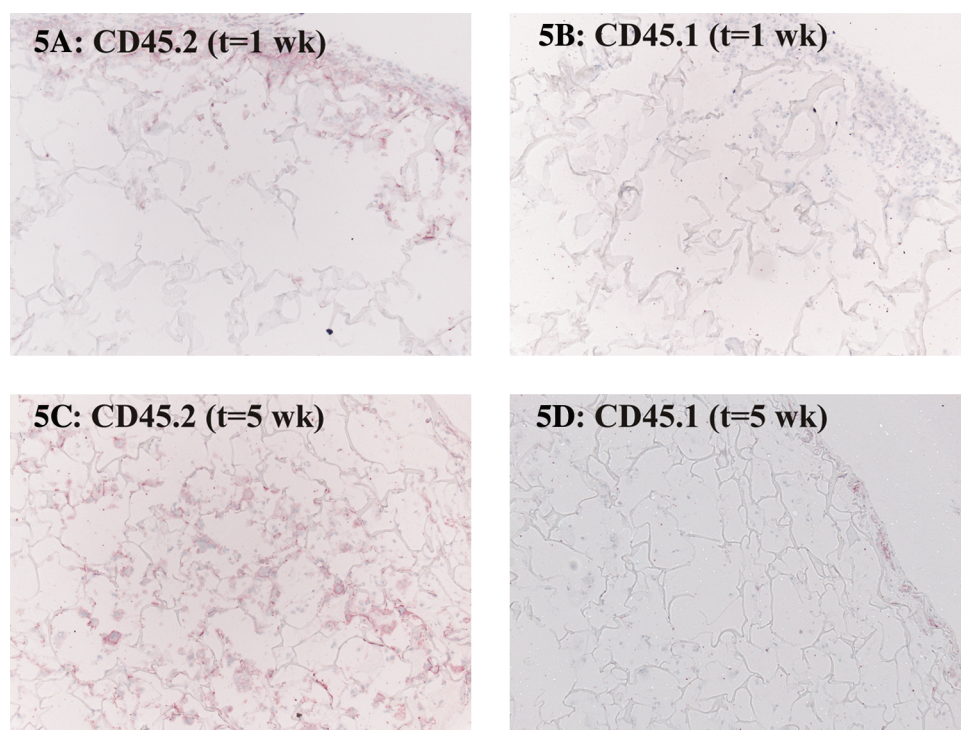
**FIG. 4.** Several cell types populate heparin-collagen scaffolds with stromal cell-derived factor 1 alpha (SDF1 $\alpha$ ) are upon implantation. Heparin-collagen scaffolds were pre-incubated with SDF1 $\alpha$  before implantation. After 5 weeks, scaffolds were collected, and sections were stained for CD11b (**A**), CD11c (**B**), or CD31 (**D**). The section in panel (**C**) was triple stained for CD150, CD41, and CD48 to detect hematopoietic stem cells. Sections were stained for the various cell markers, as described in Materials and Methods, and counterstained with hematoxylin. Original magnification:  $\times 400$  (**A**, **B**);  $\times 630$  (**C**, **D**). Experiment was performed twice in duplicate. Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

it.<sup>33</sup> However, in our scaffolds, hardly any T-cells were found, although CD31<sup>+</sup> endothelial cells were present. SDF1 $\alpha$  that remains bound to the scaffold is supposed to retain cells in the scaffold, analogous to the involvement of SDF1 in retention of hematopoietic cells within bone marrow.<sup>34,35</sup>

HPCs are attracted within 1 week, and 5 weeks after implantation, several cell types, such as CD11b<sup>+</sup> and CD11c<sup>+</sup>

myeloid cells and ERTR7<sup>+</sup> fibroblasts, populate scaffolds. Although heparin is needed to better bind SDF1 $\alpha$  to collagen, it does not have a considerable effect on cell recruitment.<sup>18</sup>

SDF1 $\alpha$ 's receptor, CXCR4, which several cell types, including HSCs and myeloid cells, B-lineage precursors and plasma cells,<sup>20</sup> and granulocytic precursors, express, mediate its effects.<sup>29</sup> Therefore, direct attraction of circulating monocytes and HSCs by SDF1 $\alpha$ , which diffuses out of those



**FIG. 5.** Cells recruited early after implantation mainly determine the cell population present in heparin-collagen scaffolds after 5 weeks. Heparin-collagen scaffolds were implanted in C57Bl6.SJL-CD45.2 mice. After 1 week, some scaffolds were collected for analysis (t = 1 wk; **A**, **B**), and others were transplanted to C57Bl6.SJL-CD45.1 mice. After another 4 weeks, scaffolds were collected (t = 5 wk; **C**, **D**). Sections of scaffolds were stained with anti-CD45.2 monoclonal antibody (mAb) 104 (**A**, **C**) or anti-CD45.1 mAb A20 (**B**, **D**), as described in Materials and Methods, and counterstained with hematoxylin. Original magnification:  $\times 100$ . One out of three experiments is shown. Each experiment was performed in duplicate. Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

scaffolds can easily explain the presence of CD11b<sup>+</sup> and CD11c<sup>+</sup> myeloid cells and CD150<sup>+</sup>CD41<sup>+</sup>CD48<sup>+</sup> HSCs in scaffolds (Fig. 4) 5 weeks after implantation, although it is expected that most circulating cells are recruited during the first 24 h after implantation, when SDF1 $\alpha$  can still form a gradient. Therefore, scaffolds should be already considerably populated by cells within 1 week after implantation, but this was not observed. On the contrary, only a few cells were present in scaffolds at that time. Apparently, those cells need inflammatory cytokines to leave the circulation and to end up in the scaffold.

After, 3D heparin–collagen scaffolds are implanted into C57Bl6 mice, several types of hematopoietic and stromal cells populate the scaffolds. Incubation with SDF1 $\alpha$  just before implantation stimulates the population of these scaffolds by cells, as long as SDF1 $\alpha$  can form a gradient.

Alternatively, therefore, we hypothesize that, instead of a number of already differentiated cells, SDF1 $\alpha$  chemo-attracts only a few HPCs in the first days after implantation. These HPCs will subsequently generate their own micro-environment and, dependent on the micro-environment they reside in, will proliferate and differentiate along different lineages, yielding a scaffold containing several cell types at 5 weeks after implantation. In agreement with this, 4 weeks after transplantation of a scaffold containing only a few CD45.2<sup>+</sup> cells to a CD45.1<sup>+</sup> environment, a substantially higher number of CD45.2<sup>+</sup> cells was present in the scaffold (Fig. 5). These cells can only have been derived from progenitor cells already present at the time of transplantation and not have been recruited in the weeks thereafter. At the time of retransplantation, an SDF1 $\alpha$  gradient does not exist anymore. Still, despite of lack of this gradient, CD45.1<sup>+</sup> cells were recruited toward the scaffold in the weeks after transplantation. The local production and secretion of chemokines and other attracting factors by cells already residing in the scaffold may explain this. No CD150<sup>+</sup>CD41<sup>+</sup>CD48<sup>+</sup> HSCs could be detected in sections of scaffolds 1 week after implantation, reflecting the limited numbers of circulating HSCs of this subset in mice. CD150<sup>+</sup>CD41<sup>+</sup>CD48<sup>+</sup> HSCs have been reported to represent only 0.0065% or less of cells in HSC-harboring organs like normal bone marrow and cyclophosphamide/granulocyte colony-stimulating factor–mobilized spleen.<sup>5</sup>

## Summary

A release of SDF1 $\alpha$  recruited hematopoietic progenitor cells toward heparinized 3D collagen scaffolds. Such scaffolds support the attracted HPCs to form their own micro-environment and develop along several lineages and attract other cells. Knowledge about the requisite factors for attraction and supporting growth and controlled differentiation of HPCs within scaffolds is of utmost importance for engineering smart scaffolds without the need for seeding the scaffolds with cells before implantation.

## Acknowledgments

We thank Dr. R. van der Voort for kindly providing the C57Bl6.SJL-CD45.1 mice and Herman van Moerkerk for preparation of collagen scaffolds. This study was financially supported by the Technology Foundation STW (grant NGC5926) and the Dutch Program for Tissue Engineering (DPTE grant 6719).

## Disclosure Statement

No competing financial interests exist.

## References

1. Wu, K., Liu, Y.L., Cui, B., and Han, Z. Application of stem cells for cardiovascular grafts tissue engineering. *Transpl Immunol* **16**, 1, 2006.
2. van Laake, L.W., Hassink, R., Doevendans, P.A., and Mummery, C. Heart repair and stem cells. *J Physiol* **577**, 467, 2006.
3. Becker, C., and Jakse, G. Stem cells for regeneration of urological structures. *Eur Urol* **51**, 1217, 2007.
4. Horch, R.E. Future perspectives in tissue engineering. *J Cell Mol Med* **10**, 4, 2006.
5. Kiel, M.J., Yilmaz, O.H., Iwashita, T., Yilmaz, O.H., Terhorst, C., and Morrison, S.J. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109, 2005.
6. Yilmaz, O.H., Kiel, M.J., and Morrison, S.J. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood* **107**, 924, 2006.
7. Hess, D.A., Wirthlin, L., Craft, T.P., Herrbrich, P.E., Hohm, S.A., Lahey, R., Eades, W.C., Creer, M.H., and Nolte, J.A. Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. *Blood* **107**, 2162, 2006.
8. Wilson, A., and Trumpp, A. Bone-marrow hematopoietic-stem-cell niches. *Nat Rev Immunol* **6**, 93, 2006.
9. Nagasawa, T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol* **6**, 107, 2006.
10. Adams, G.B., and Scadden, D.T. The hematopoietic stem cell in its place. *Nat Immunol* **7**, 333, 2006.
11. Taichman, R.S. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood* **105**, 2631, 2005.
12. Whetton, A.D., and Graham, G.J. Homing and mobilization in the stem cell niche. *Trends Cell Biol* **9**, 233, 1999.
13. Bleul, C.C., Fuhlbrigge, R.C., Casasnovas, J.M., Aiuti, A., and Springer, T.A. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* **184**, 1101, 1996.
14. Netelenbos, T., Zijderduijn, S., van den, B.J., Kessler, F.L., Zweegman, S., Huijgens, P.C., and Drager, A.M. Proteoglycans guide SDF-1-induced migration of hematopoietic progenitor cells. *J Leukoc Biol* **72**, 353, 2002.
15. Aiuti, A., Webb, I.J., Bleul, C., Springer, T., and Gutierrez-Ramos, J.C. The chemokine SDF-1 is a chemoattractant for human CD34<sup>+</sup> hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34<sup>+</sup> progenitors to peripheral blood. *J Exp Med* **185**, 111, 1997.
16. Wright, D.E., Bowman, E.P., Wagers, A.J., Butcher, E.C., and Weissman, I.L. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. *J Exp Med* **195**, 1145, 2002.
17. Lapidot, T., Dar, A., and Kollet, O. How do stem cells find their way home? *Blood* **106**, 1901, 2005.
18. Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K., and Nagasawa, T. Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. *Immunity* **19**, 257, 2003.

19. Nimura, F., Zhang, L.F., Okuma, K., Tanaka, R., Sunakawa, H., Yamamoto, N., and Tanaka, Y. Cross-linking cell surface chemokine receptors leads to isolation, activation, and differentiation of monocytes into potent dendritic cells. *Exp Biol Med* **231**, 431, 2006.
20. Kortessidis, A., Zannettino, A., Isenmann, S., Shi, S., Lapidot, T., and Gronthos, S. Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. *Blood* **105**, 3793, 2005.
21. Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.I., and Nagasawa, T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* **20**, 707, 2004.
22. Netelenbos, T., van den, B.J., Kessler, F.L., Zweegman, S., Merle, P.A., van Oostveen, J.W., Zwaginga, J.J., Huijgens, P.C., and Drager, A.M. Proteoglycans on bone marrow endothelial cells bind and present SDF-1 towards hematopoietic progenitor cells. *Leukemia* **17**, 175, 2003.
23. Netelenbos, T., van den, B.J., Kessler, F.L., Zweegman, S., Huijgens, P.C., and Drager, A.M. *In vitro* model for hematopoietic progenitor cell homing reveals endothelial heparan sulfate proteoglycans as direct adhesive ligands. *J Leukoc Biol* **74**, 1035, 2003.
24. Wissink, M.J., Beernink, R., Pieper, J.S., Poot, A.A., Engbers, G.H., Beugeling, T., van Aken, W.G., and Feijen, J. Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and *in vitro* evaluation. *Biomaterials* **22**, 151, 2001.
25. Hatton, M.W., Berry, L.R., Machovich, R., and Regoeczi, E. Tritiation of commercial heparins by reaction with NaB<sup>3</sup>H<sub>4</sub>: chemical analysis and biological properties of the product. *Anal Biochem* **106**, 417, 1980.
26. Pieper, J.S., Oosterhof, A., Dijkstra, P.J., Veerkamp, J.H., and van Kuppevelt, T.H. Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate. *Biomaterials* **20**, 847, 1999.
27. Wang, C.L., Miyata, T., Weksler, B., Rubin, A.L., and Stenzel, K.H. Collagen-induced platelet aggregation and release. I Effects of side-chain modifications and role of arginyl residues. *Biochim Biophys Acta* **544**, 555, 1978.
28. Loke, W.K., and Khor, E. Validation of the shrinkage temperature of animal tissue for bioprosthetic heart valve application by differential scanning calorimetry. *Biomaterials* **16**, 251, 1995.
29. Markwell, M.A. A new solid-state reagent to iodinate proteins. I. Conditions for the efficient labeling of antiserum. *Anal Biochem* **125**, 427, 1982.
30. Gupta, P., Oegema, T.R., Jr., Brazil, J.J., Dudek, A.Z., Slungaard, A., and Verfaillie, C.M. Structurally specific heparan sulfates support primitive human hematopoiesis by formation of a multimolecular stem cell niche. *Blood* **92**, 4641, 1998.
31. Diamond, M.S., Alon, R., Parkos, C.A., Quinn, M.T., and Springer, T.A. Heparin is an adhesive ligand for the leukocyte integrin Mac-1 (CD11b/CD1). *J Cell Biol* **130**, 1473, 1995.
32. den Dekker, E., Grefte, S., Huijs, T., Versteeg, E.M.M., Bladergroen, B.A., van Kuppevelt, T.H., Figdor, C.G., and Torensma, R. Monocyte cell surface GAGs positively modulate IL-4-induced differentiation towards DCs. *J Immunol* **180**, 3680, 2008.
33. Cinamon, G., Shinder, V., and Alon, R. Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. *Nat Immunol* **2**, 515, 2001.
34. Lapidot, T., and Petit, I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp Hematol* **30**, 973, 2002.
35. Ma, Q., Jones, D., and Springer, T.A. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* **10**, 463, 1999.

Address correspondence to:

Ruurd Torensma, Ph.D.

Department of Tumor Immunology, NCMLS 278

Radboud University Nijmegen Medical Centre

Geert Grooteplein 28

6525 GA Nijmegen

The Netherlands

E-mail: r.torensma@ncmls.ru.nl

Received: June 20, 2008

Accepted: October 15, 2008

Online Publication Date: December 18, 2008

